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TITLE:	Glycated insulin as a biomarker for diagnosis and monitoring of diabetes.		

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DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
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Sir:

I, Peter Raymond Flatt, declare as follows:

1. I am named as one of the inventors in the above-identified application.
2. I have been working in the field of diabetes research for more than 30 years.
3. Obese-hyperglycemic ob/ob mice lack functional leptin. Ob/ob mice are grossly overweight, particularly at young ages, and develop severe insulin resistance. They are widely used as a model for obesity and diabetes.
4. Aston ob/ob mice are a strain of ob/ob mice that have severe insulin resistance and are also hyperglycaemic. Aston ob/ob mice are widely used as a model for obesity and diabetes. The derivation and characteristics of this animal model have been previously described (Flatt P R, Bailey C J, Development of glucose intolerance and impaired plasma insulin response to glucose in obese hyperglycaemic (ob/ob) mice. Horm. Metab. Res. 1981 ; 13 : 556-560, and Bailey CJ, Flatt PR & Atkins TW, Influence of genetic background and age on expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. International Journal of Obesity 6: 11-21; 1982).

5. I declare that Aston ob/ob mice develop obesity before 3 weeks of age and are known from the literature to exhibit hyperglycaemia after 7 weeks of age (see Bailey CJ, Flatt PR & Atkins TW, Influence of genetic background and age on expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. International Journal of Obesity 6: 11-21; 1982).

6. In Aston ob/ob mice, glucose levels are normal at 6 weeks of age, but are raised at 9 weeks of age, when compared with normal control mice (see attached Figure).

7. I declare that Aston ob/ob mice are in a pre-diabetic state at 6 weeks of age but have developed diabetes by 9 weeks of age.

8. In Aston ob/ob mice, glycated insulin levels are raised at 6 weeks of age, compared with normal control mice. At 9 weeks, glycated insulin levels, although still raised, are lower than glycated insulin levels at 6 weeks of age (see attached Figure). These data support our submissions that glycated insulin levels fall off after diabetes is diagnosed, so that glycated insulin levels are most raised just before and around diabetes onset.

9. Glycated insulin levels are raised at 6 weeks of age and this is prior to the later onset of hyperglycaemia at 9 weeks (see attached Figure). I declare that glycated insulin levels are raised in the pre-diabetic state in Aston ob/ob mice.

10. Under my direction and control, plasma glucose concentrations were measured in *ad libitum* fed mice using an analyser based on the glucose oxidase technique. Plasma glycated insulin was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) with luminescence detection. All measurements were made using fed mice.

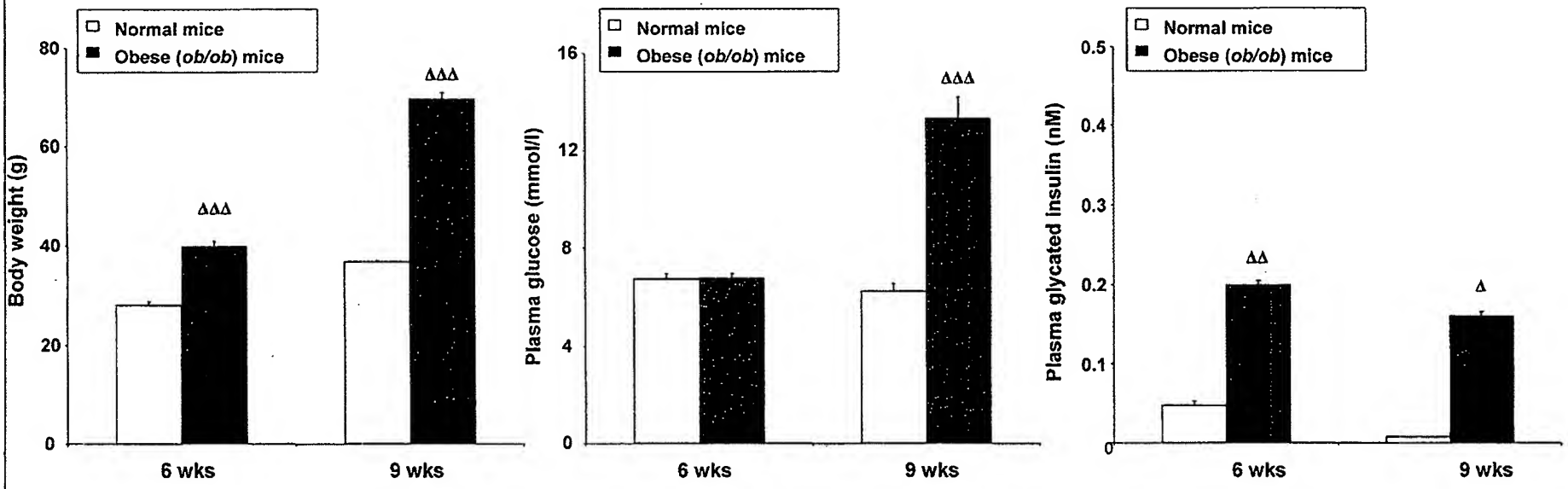
11. I declare that these data support the teaching of our Patent Specification in demonstrating that the methods and uses as claimed can be used to effectively diagnose early diabetes and / or determine a predisposition to diabetes in an individual.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

11/2/09
Date

P R Flatt
Peter Raymond Flatt

Elevation of plasma glycated insulin concentrations in 6 week old *ob/ob* mice precedes the later development of hyperglycaemia at 9 weeks



Values are Mean±SEM (n=6) $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ and $^{\Delta\Delta\Delta}P<0.001$, compared with normal mice

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Development of Glucose Intolerance and Impaired Plasma Insulin Response to Glucose in Obese Hyperglycaemic (ob/ob) Mice

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Summary

The development of glucose intolerance in Aston ob/ob mice showed a gross exaggeration of the age-related changes of glucose tolerance in lean (+/+) mice. Intraperitoneal glucose tolerance in ob/ob mice was poor at 5 weeks, improved by 10 weeks, but markedly worsened by 20 weeks. A 24 hour fast further exaggerated the glucose intolerance of ob/ob mice. Unlike lean mice, tolerance improved in ob/ob mice at 40 weeks. Alterations of insulin sensitivity and the plasma insulin response to glucose accounted in part for these observations. Insulin sensitivity deteriorated until 20 weeks, but improved at 40 weeks in both fed and 24 hour fasted ob/ob mice. A positive plasma insulin response to glucose was lost after 5 weeks in fed ob/ob mice. The severity of this abnormality corresponded with the extent of the basal hyperinsulinaemia. A 24 hour fast reduced plasma insulin concentrations and restored a positive plasma insulin response to glucose in ob/ob mice. The results suggest that the plasma insulin response to glucose in ob/ob mice is related to the secretory activity of the B-cells prior to stimulation. Furthermore, it is evident that factors in addition to insulin insensitivity and the impaired plasma insulin response to glucose contribute to the development of glucose intolerance in these mice.

Key-Words: *Glucose Tolerance — Plasma Insulin — Insulin Sensitivity — Obese Hyperglycaemic (ob/ob) Mice*

Introduction

Freely fed adult obese hyperglycaemic (ob/ob) mice exhibit an impaired insulin response to an intraperitoneal or intravenous glucose challenge. Indeed, these mice often respond to glucose with a fall in the plasma insulin concentration (*Genuth* 1969; *Herberg, Major, Hennigs, Grüneklee, Freytag and Gries* 1970; *Westman* 1970; *Beloff-Chain, Freund and Rookledge* 1975). Also, glucose tolerance is impaired and there is marked insensitivity to insulin (*Westman* 1968; *Chlouverakis and White* 1969; *Stauffacher and Renold* 1969; *Abraham and Beloff-Chain* 1971; *Stauffacher, Orci, Cameron, Burr and Renold* 1971; *Cuendet, Loten, Jeanrenaud and Renold* 1976).

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The present study describes the development of glucose intolerance and the impaired plasma insulin response to glucose in Aston ob/ob mice. We demonstrate the dependence of these features on nutritional status and on the extent of hyperinsulinaemia prior to glucose administration. In addition, we compare the development of abnormal glucose tolerance and plasma insulin concentrations in ob/ob mice with the age-related changes in homozygous lean (+/+) mice.

Materials and Methods

Obese hyperglycaemic (ob/ob) mice and homozygous lean (+/+) mice from the colony maintained at the University of Aston in Birmingham were used. Expression of the ob gene on this background results in a more severe form of diabetes than C57BL/6J ob/ob mice (*Herberg and Coleman* 1977; *Bailey, Flatt and Atkins* 1981). The origin and characteristics of Aston ob/ob mice have been described in detail elsewhere (*Bailey, Flatt and Atkins* 1981; *Flatt and Bailey* 1981). Control mice were taken from an established true breeding line of homozygous lean (+/+) mice because heterozygous lean (ob/+) mice exhibit a partial expression of the obese hyperglycaemic syndrome (*Flatt and Bailey* 1981). Mice were housed in an air-conditioned room at 22 ± 2 °C with a lighting schedule of 9.5 hours light (0800–1730) and 14.5 hours dark. A standard pellet diet (Mouse breeding diet, Heygate & Sons Ltd., Northampton, U.K.) and water were supplied ad libitum.

Intraperitoneal glucose tolerance tests and insulin hypoglycaemia tests were performed at 0900 hours on freely fed and 24 hour fasted mice at ages selected from 5 to 40 weeks. Food and water were withheld during the tests. Blood samples (50 µl) were collected from the cut tip of the tail of conscious mice immediately before injection of either glucose (2 g/kg in a 40% w/v solution) or monocomponent porcine insulin (Actrapid®, 0.25, 5, 50 or 100 U/5 ml/kg). Subsequent blood samples were collected at 30 and 60 minutes after glucose, and 15, 30 and 60 minutes after insulin administration. The mice were previously adapted to the manipulative procedures, and preliminary experiments established that intraperitoneal injection of saline (5 ml/kg) did not affect plasma glucose and insulin concentrations. Previous experiments revealed no effect of the blood sampling procedure on the plasma glucagon concentration (*Flatt, Bailey and Buchanan* 1980).

Plasma was separated and stored at −20 °C. Plasma glucose was determined by an automated glucose oxidase procedure (Beckman Glucose Analyzer, Beckman Riic Ltd., High Wycombe, U.K.) (*Stevens* 1971), and plasma insulin was measured by dextran-charcoal radioimmunoassay (*Albano, Ekins, Maritz and Turner* 1972) using crystalline mouse insulin as standard (Novo Industria A/S, Copenhagen).

Statistical evaluation of the results was performed using Student's t-test. Differences were considered to be significant for $P < 0.05$.

Results

Glucose tolerance and plasma insulin (Table 1)

Fed lean mice. Basal plasma glucose concentrations in fed lean mice were not significantly altered between 5 and 40 weeks. Glucose tolerance was impaired at 5 weeks, but there were no significant differences between 10, 20 and 40 weeks. Basal and glucose-stimulated plasma insulin concentrations were lowest at 10 weeks. A marked but transient insulin response to glucose occurred at 5 weeks.

Fasted lean mice. In 24 hour fasted lean mice, plasma glucose concentrations in the basal state and during glucose tolerance tests were highest at 5 weeks, reduced at 10 weeks, but increased between 10 and 40 weeks. Similarly, basal and glucose-stimulated plasma insulin concentrations were raised at 5 weeks, reduced at 10 weeks, but increased again by 40 weeks.

Fed obese mice. At 5 weeks the basal plasma glucose concentration in fed obese mice was not significantly greater than fed lean mice, but a moderate hyperglycaemia was observed after 8 weeks. Values at 40 weeks were not significantly greater than lean mice. Glucose tolerance was impaired in fed obese mice at all ages studied. Tolerance was poor at 5 weeks, but improved at 8 and 10 weeks. At 15 and 20 weeks tolerance became progressively worse, while at 40 weeks a considerable improvement was observed. A marked basal hyperinsulinaemia was noted in fed obese mice at each age. The plasma insulin concentration rose sharply from 5 to 10 weeks, and declined progressively thereafter. At 5 weeks intraperitoneal glucose produced a large but transient positive insulin response. However, in older mice glucose failed to evoke a significant rise in plasma insulin. As the basal insulin concentration increased, the positive aspect of the response became reduced, and at 10 weeks, when the basal insulin concentration was maximal, glucose produced a considerable fall in the plasma insulin concentration. At 15, 20 and 40 weeks, when the basal insulin concentrations were lower, glucose produced a less marked fall in the insulin concentration, but a significant positive insulin response was not observed.

Fasted obese mice. In 24 hour fasted obese mice, the basal plasma glucose concentration was significantly higher than fasted lean mice at 5, 10 and 20 weeks, but values at 40 weeks not significantly different. Glucose tolerance was impaired at all ages. Tolerance was poor at 5 weeks, improved by 10 weeks, but deteriorated at 20 weeks. A small improvement was noted at 40 weeks. The basal plasma insulin concentration and the insulin response to glucose in fasted obese mice were greater than fasted lean mice at 10, 20 and 40 weeks, but not at 5 weeks.

In contrast to fed obese mice, the fasted obese mice showed a positive insulin response to glucose at all ages.

Insulin hypoglycaemia tests (Table 2)

Insulin insensitivity was established in fed obese mice at 5 weeks as shown by an impaired hypoglycaemic effect of 0.25 U/kg insulin. Insulin sensitivity became severely impaired at 10 and 20 weeks. This is demonstrated by the requirement for 100 U/kg insulin to achieve a rate of glucose disappearance approaching that produced by 5 U/kg in the 5 weeks old obese mice. Insulin sensitivity was considerably improved at 40 weeks, when the hypoglycaemic effect of insulin was comparable with 5 weeks old obese mice. Insulin sensitivity was better in 24 hour fasted mice than fed mice at all ages except 40 weeks.

Discussion

Glucose homeostasis in lean (+/+) mice was poor at 5 weeks, improved at 10 weeks but deteriorated in older mice. The impaired glucose tolerance and transient insulin response at 5 weeks might reflect a continuing influence of the metabolic bias to prevent hypoglycaemia in early life (*Adam* 1971). Furthermore, the transition at weaning (3 weeks of age) from a restricted milk diet to a solid diet, richer in carbohydrate and available ad libitum may create a temporary imbalance in plasma glucose regulation (*Dubuc* 1976). Insulin sensitivity was not reduced at this age, but

Table 1 Plasma glucose and insulin concentrations during intraperitoneal glucose tolerance tests at different ages in freely fed and 24 hour

Gene type and nutritional status	Age (weeks)	Body weight (g)	Plasma glucose (mmol/l)				
			0 min	30 min	60 min	Total$^{\Phi}$	Increase$^{\#}$
Lean (+/+) fed	5	22.3 ± 0.5	7.8 ± 0.3	11.8 ± 1.1^{c}	11.1 ± 0.5^{cef}	30.9 ± 1.2^{cef}	7.3 ± 1.1^{cef}
	10	25.5 ± 0.6	7.3 ± 0.2	9.6 ± 0.3^{a}	8.8 ± 0.7^{a}	25.7 ± 0.6^{a}	3.7 ± 0.5^{a}
	20	31.6 ± 1.1	7.3 ± 0.5	10.5 ± 1.0	8.8 ± 0.5^{a}	26.7 ± 0.9^{a}	4.6 ± 0.7^{a}
	40	33.5 ± 1.3	7.4 ± 0.4	10.5 ± 0.9	8.9 ± 0.3^{a}	26.9 ± 0.8^{a}	4.6 ± 0.7^{a}
Lean (+/+) 24 h fasted	5	21.8 ± 0.4	4.8 ± 0.2^{c}	20.5 ± 1.2^{cef}	16.2 ± 1.0^{cef}	41.6 ± 1.3^{cef}	27.1 ± 1.3^{cef}
	10	25.1 ± 0.6	3.9 ± 0.2^{af}	14.0 ± 1.1^{a}	9.8 ± 0.5^{af}	27.8 ± 1.0^{af}	16.0 ± 0.9^{af}
	20	31.3 ± 1.0	4.3 ± 0.1	12.2 ± 0.8^{af}	11.3 ± 0.7^{a}	28.0 ± 1.0^{af}	14.9 ± 1.0^{af}
	40	32.2 ± 1.1	4.5 ± 0.2^{c}	17.0 ± 1.7^{ae}	12.6 ± 1.1^{ac}	34.2 ± 1.8^{ace}	20.6 ± 1.8^{ace}
Obese (ob/ob) fed	5	28.9 ± 2.5	8.6 ± 0.6^{cde}	25.8 ± 2.0^{+}	25.2 ± 0.8^{+cf}	59.7 ± 1.7^{+bcef}	33.9 ± 1.6^{+bcdf}
	8	49.9 ± 3.4	9.7 ± 1.0	21.7 ± 1.1^{e}	20.7 ± 2.9	52.3 ± 2.3^{a}	20.0 ± 2.1^{ae}
	10	61.1 ± 4.1	10.8 ± 0.7^{+a}	20.3 ± 2.5^{+e}	18.0 ± 2.6^{+ade}	49.3 ± 3.3^{+ade}	16.6 ± 3.2^{+ade}
	15	72.8 ± 5.5	11.4 ± 0.7^{af}	23.2 ± 2.6^{e}	24.1 ± 1.7^{cf}	58.8 ± 2.7^{cef}	24.5 ± 2.6^{ace}
	20	79.8 ± 6.9	11.1 ± 0.3^{+af}	29.6 ± 1.0^{+bcdf}	25.2 ± 2.6^{+cf}	66.0 ± 2.1¶^{acd}	32.6 ± 2.1¶^{bcdf}
	40	89.3 ± 10.4	8.7 ± 1.1^{de}	19.0 ± 2.8^{+e}	17.8 ± 2.7^{+ade}	45.6 ± 3.5^{+ad}	19.3 ± 3.1^{+ae}
Obese (ob/ob) 24 h fasted	5	27.2 ± 2.2	6.5 ± 0.5^{*cef}	25.3 ± 1.9^{*ce}	26.3 ± 2.6^{+ce}	58.2 ± 2.6^{+ce}	38.7 ± 2.4^{+cef}
	10	48.3 ± 3.1	5.5 ± 0.4^{+ae}	18.6 ± 1.8^{*aef}	20.6 ± 1.7^{+aef}	44.8 ± 2.3^{+aef}	28.1 ± 2.3^{+aef}
	20	60.7 ± 4.5	8.7 ± 0.5^{+acf}	32.8 ± 1.3^{+ac}	37.1 ± 1.7¶^{acf}	78.7 ± 2.0¶^{acf}	52.5 ± 2.0¶^{ac}
	40	89.0 ± 11.3	4.6 ± 0.6^{ae}	31.7 ± 3.1^{+c}	27.6 ± 2.1^{+ce}	64.0 ± 3.1¶^{ce}	50.0 ± 2.9¶^{ac}

$^{\Phi}$Total is the sum of values at 0, 30 and 60 min. $^{\#}$Increase is the sum of values at 30 and 60 min minus twice the value at zero time. ^{abcdef}Superscript letters indicate $P < 0.05$ compared with ^{a}5 weeks, ^{b}8 weeks, ^{c}10 weeks, ^{d}15 weeks, ^{e}20 weeks, ^{f}40 weeks of age for the ln 24 h fasted lean (+/+) mice the increase in plasma glucose was greater ($P < 0.05$) and the total plasma insulin was smaller ($P < 0.05$) than ln 24 h fasted obese (ob/ob) mice the increase in plasma glucose was greater ($P < 0.05$) than fed ob/ob mice at 10, 20 and 40 weeks of age.

Table 2 Plasma glucose concentrations at different ages in freely fed and 24 hour fasted lean (+/+) and obese (ob/ob) mice after intraperitoneal administration of insulin. Values are mean ± SEM of 6–10 determinations

Age (weeks)	Insulin dose (U/kg)	Nutritional status	Body weight (g)	Plasma glucose (mmol/l)				t 1/2 glucose (min)*	Rate of glucose disappearance (%/min)$^{+}$
				0 min	15 min	30 min	60 min		
Lean (+/+)									
5	0.25	fed	22.4 ± 0.9	7.8 ± 0.3	4.2 ± 0.2	2.7 ± 0.2	2.7 ± 0.3	19.7 ± 1.3^{a}	3.52 ± 0.29^{b}
Obese (ob/ob)									
5	0.25	fed	26.8 ± 1.3	8.5 ± 0.7	8.3 ± 0.6	6.4 ± 1.1	8.4 ± 0.6	93.9 ± 21.6^{a}	1.02 ± 0.32^{b}
5	5	fed	26.5 ± 1.4	8.0 ± 0.5	5.0 ± 0.3	3.8 ± 0.3	4.6 ± 0.3	29.5 ± 4.6	2.46 ± 0.36^{g}
5	5	24 h fasted	26.3 ± 2.0	6.1 ± 0.4	3.5 ± 0.2	1.7 ± 0.2	1.4 ± 0.1	16.8 ± 2.5	4.16 ± 0.28
5	50	fed	27.2 ± 2.0	8.0 ± 0.2	3.7 ± 0.3	2.7 ± 0.2	2.7 ± 0.3	19.4 ± 1.3^{c}	3.59 ± 0.23^{d}
5	50	24 h fasted	26.8 ± 1.5	6.3 ± 0.7	1.5 ± 0.2	1.0 ± 0.1	0.7 ± 0.1	12.2 ± 1.9^{h}	5.92 ± 0.79^{i}
10	50	fed	60.0 ± 7.0	10.8 ± 0.6	9.1 ± 0.3	7.6 ± 0.2	9.0 ± 0.4	60.4 ± 5.3^{c}	1.16 ± 0.09^{d}
10	50	24 h fasted	54.0 ± 5.3	5.6 ± 0.6	4.1 ± 0.1	2.5 ± 0.3	1.1 ± 0.1	28.8 ± 5.7^{h}	2.67 ± 0.46^{i}
10	100	fed	60.2 ± 2.4	11.4 ± 0.6	7.8 ± 1.0	6.4 ± 0.6	5.5 ± 0.5	41.9 ± 8.0	1.83 ± 0.33
10	100	24 h fasted	56.2 ± 2.3	5.9 ± 0.5	2.7 ± 0.3	1.2 ± 0.1	1.0 ± 0.1	13.8 ± 1.6^{e}	5.19 ± 0.54^{f}
20	100	fed	85.0 ± 3.1	11.5 ± 0.3	9.0 ± 0.2	8.1 ± 0.2	7.6 ± 0.5	55.5 ± 5.2	1.25 ± 0.30
20	100	24 h fasted	83.0 ± 2.8	8.5 ± 0.5	6.0 ± 0.5	3.9 ± 0.3	2.5 ± 0.3	27.6 ± 2.1^{e}	2.56 ± 0.16^{f}
40	5	fed	88.7 ± 7.5	9.0 ± 1.0	6.1 ± 0.5	3.3 ± 0.3	3.5 ± 0.4	20.9 ± 3.5	3.32 ± 0.34^{g}
40	5	24 h fasted	85.8 ± 7.3	5.0 ± 0.6	3.2 ± 0.4	1.6 ± 0.2	1.2 ± 0.2	18.3 ± 2.6	3.79 ± 0.29

*t 1/2 for plasma glucose is the time taken in minutes for the plasma glucose concentration to fall to half of the concentration at zero time. The t 1/2 was calculated from the plasma glucose concentrations at 0 and 30 min plotted on a logarithmic scale against time.

$^{+}$Rate of glucose disappearance was calculated from the equation $\frac{\log_e 2 \times 100}{t\ 1/2}$ using the t 1/2 value derived as above.

$^{abcdef}p < 0.01$, $^{ghip} < 0.05$ compared with group bearing the same superscript letter.

For each dose of insulin, the t 1/2 for glucose was smaller ($P < 0.05$) and the rate of glucose disappearance was greater ($P < 0.05$) in 24 hour fasted mice than fed mice at all ages except 40 weeks.

a deterioration in older mice (*Bailey and Flatt*, unpublished) may contribute to the impaired glucose tolerance at 40 weeks. As expected, a 24 hour fast reduced glucose tolerance and suppressed the plasma insulin response to glucose in lean mice at all ages (*Grey, Goldring and Kipnis* 1970; *Malaisse* 1972; *Hedeskov and Capito* 1974).

fasted lean (+/+) and obese (ob/ob) mice. Values are mean ± SEM of 6–10 determinations

Plasma insulin (ng/ml)				
0 min	30 min	60 min	Total$^{\Phi}$	Increase$^{\neq}$
2.0 ± 0.3^{c}	5.2 ± 1.8^{c}	1.6 ± 0.6^{ef}	8.8 ± 1.7^{c}	2.8 ± 1.3
0.9 ± 0.3^{aef}	1.6 ± 0.6^{aef}	1.4 ± 0.3^{ef}	3.9 ± 0.6^{aef}	1.2 ± 0.4^{ef}
2.1 ± 0.5^{c}	4.4 ± 0.7^{c}	3.1 ± 0.5^{ac}	9.6 ± 0.8^{c}	3.3 ± 0.6^{c}
2.6 ± 0.5^{c}	4.6 ± 0.6^{c}	3.5 ± 0.6^{ac}	10.7 ± 0.9^{c}	2.9 ± 0.7^{c}
1.1 ± 0.2^{ce}	2.5 ± 0.3^{cef}	1.1 ± 0.2	4.7 ± 0.4^{ce}	1.4 ± 0.3^{f}
0.4 ± 0.1^{af}	1.3 ± 0.2^{a}	0.7 ± 0.2	2.4 ± 0.2^{af}	1.2 ± 0.2^{f}
0.5 ± 0.1^{af}	1.5 ± 0.3^{a}	0.7 ± 0.2	2.7 ± 0.3^{af}	1.2 ± 0.3
1.2 ± 0.2^{ce}	1.6 ± 0.3^{a}	1.2 ± 0.2	4.0 ± 0.4^{ce}	0.4 ± 0.3^{ac}
11.5 ± 4.3^{+cde}	24.7 ± 5.7^{+}	6.7 ± 2.9^{+de}	42.9 ± 6.8^{+cde}	8.4 ± 5.9^{*bcd}
17.6 ± 3.4	18.5 ± 3.8	9.8 ± 3.1^{de}	45.9 ± 4.9^{cde}	−6.9 ± 4.1^{ac}
32.3 ± 10.0^{+a}	22.0 ± 5.4¶	12.1 ± 4.6^{+e}	66.4 ± 10.3¶^{abf}	−30.5 ± 6.3^{*abd}
26.5 ± 6.7^{a}	23.6 ± 4.8	20.5 ± 3.7^{ab}	70.6 ± 7.2^{abf}	−8.9 ± 5.2^{ac}
24.0 ± 7.4^{+a}	23.2 ± 6.6^{+}	26.6 ± 3.2¶^{abcf}	73.8 ± 7.5¶^{abf}	1.8 ± 5.1
15.1 ± 4.3^{+}	14.0 ± 3.7^{+}	13.5 ± 4.2^{+e}	42.6 ± 6.1^{+cde}	2.7 ± 4.7
1.3 ± 0.2^{cef}	2.6 ± 0.4^{cef}	1.8 ± 0.3^{cef}	5.7 ± 0.4^{cef}	1.7 ± 0.4^{f}
3.6 ± 0.6^{+ae}	5.9 ± 0.7^{+a}	3.5 ± 0.5^{+aef}	13.0 ± 0.9^{+ae}	2.2 ± 0.6^{f}
5.6 ± 0.7^{+acf}	7.2 ± 1.0^{+a}	5.6 ± 0.6¶^{ac}	18.4 ± 1.5^{+acf}	1.6 ± 0.9^{f}
2.5 ± 0.4^{+ae}	6.5 ± 1.3^{+a}	5.5 ± 1.1^{+ac}	14.5 ± 1.7^{+ae}	7.0 ± 1.5^{*ace}

$^{*}P < 0.05$, $^{+}P < 0.01$, ¶$P < 0.001$ compared with +/+ mice of the same age and nutritional status.
same gene type and same nutritional status.
fed +/+ mice of the same age.

The changes in glucose tolerance during the development of the ob/ob syndrome reflected the age-related pattern in lean mice. Thus, tolerance was poor at 5 weeks, improved at 8 and 10 weeks but markedly worsened by 20 weeks. However, in contrast to lean mice, tolerance improved in obese mice at 40 weeks, especially in the fed state. The reduced sensitivity to insulin at different stages of the syndrome cannot account fully for the changes in glucose tolerance. The impaired tolerance at 5 weeks and the improvement at 40 weeks corresponded with appropriate changes of insulin sensitivity. However, the improvement of glucose tolerance between 5 and 10 weeks was coexistent with a considerable deterioration of insulin sensitivity.

Furthermore, fasting impaired glucose tolerance but improved insulin sensitivity. The changes of insulin sensitivity at different ages and after fasting showed a reciprocal relationship with the prevailing insulin concentration. This is consistent with reports that insulin down regulates insulin receptors in obese mice (*Forgue and Freychet* 1975; *Soll, Kahn, Neville and Roth* 1975).

An intraperitoneal glucose challenge evoked a positive plasma insulin response at 5 weeks. However, as previously reported, glucose failed to produce a significant rise in plasma insulin concentrations of older fed obese mice (*Genuth* 1969; *Herberg* et al. 1970; *Beloff-Chain* et al. 1975). Although the glucose environment prior to stimulation can influence insulin secretion (*Andersson, Asplund and Larkins* 1978; *Gylfe* 1978), the different plasma insulin responses in the present study could not be attributed to variations in the pre-stimulatory glucose concentration. However, the response did show an inverse association with the extent of basal hyperinsulinaemia, suggesting that the insulin response is dependent on the secretory activity prior to glucose stimulation. The hyperinsulinaemia of obese mice is also associated with severe B-cell degranulation (*Herberg* et al. 1970; *Bailey, Flatt and Atkins* 1981).

Thus excessive secretory activity might impair the ability of the B-cells to recognise or respond to a glucose challenge. Accordingly, treatment of obese mice with the insulin secretagogue pilocarpine, abolished glucose-stimulated insulin secretion from islets subsequently incubated in vitro (*Atkins, Best, Flatt, Bailey and Matty* 1975). The possible depletion of labile intracellular insulin stores in fed obese mice is suggested by the observation that fasting replenishes the pancreatic insulin content (*Stauffacher, Lambert, Vecchio and Renold* 1967), and as shown in this and other studies (*Westman* 1970; *Cameron, Stauffacher, Amherdt, Orci and Renold* 1972) also restores a positive plasma insulin response to glucose. However, since arginine and glucagon evoke a marked plasma insulin response in fed obese mice (*Flatt and Bailey*, in preparation), it is unlikely that degranulation is sufficient to account for the failure of the B-cells to respond to glucose. An alternative proposition that there exists a specific defect in the recognition or stimulus-secretion coupling of glucose-induced insulin release is supported by the observation that orally administered glucose produced a marked plasma insulin response in fed obese mice (*Flatt and Bailey* 1981). Thus stimuli generated by the ingestion of glucose (*Creutzfeldt* 1979; *Bailey* 1980) appear essential for glucose stimulation of insulin release in adult fed obese mice.

In conclusion, the development of glucose intolerance in obese mice can be attributed partly to insulin insensitivity and a loss of the plasma insulin response to glucose. How-

ever, additional factors contribute to the changes of glucose tolerance in these mice.

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Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston *ob/ob* mice

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Summary
Expression of the obese hyperglycaemic *(ob/ob)* syndrome in mice is modified by the background genome. The Aston colony carries the *ob* gene on a mixed background which produces a unique combination of different features shown by *ob/ob* mice on other backgrounds. The maximum body weight of Aston *ob/ob* mice exceeded that of other colonies, possibly reflecting a trait for higher growth rate in the background genome. The hyperphagia, marked hyperinsulinaemia and moderate hyperglycaemia observed during the development of the syndrome receded in old mice. Plasma glucagon concentrations in the fed state were similar to +/+ mice and did not vary throughout life. Hyperplasia of the B-cells increased inordinately during the development of the syndrome, but declined in older mice coincident with progressive intercellular vacuolation and the appearance of acinar-like cells within the islets. A-cell hyperplasia was greater in young mice, and A-cells became relocated throughout the islets of older mice. The distinct pattern of age-related changes in *ob/ob* mice indicates that experiments using this gene type should define clearly their age as well as genetic background.

Introduction

The obese hyperglycaemic syndrome in mice (gene type *ob/ob*) is used extensively as an animal model of obesity and non-insulin-dependent diabetes mellitus[11, 33]. The syndrome is inherited as an autosomal recessive trait which is now maintained on several different genetic backgrounds.

On the C57BL/6J (BL/6) background and certain other backgrounds, the syndrome is characterized by marked obesity, mild or moderate hyperglycaemia and inordinate hyperinsulinaemia[14, 16, 33, 34, 55]. However, on the C57BL/KsJ (BL/Ks) background *ob/ob* mice show a less marked obesity, a severe and protracted hyperglycaemia, and a mild transient hyperinsulinaemia with subsequent hypoinsulinaemia[5, 16]. The islets of these mice also differ. On the BL/6 and other

backgrounds *ob/ob* mice exhibit marked hypertrophy and hyperplasia of the islets co-existent with hyperplasia and degranulation of the B-cells[5, 8, 9, 16, 25, 31, 34, 56]. In BL/Ks *ob/ob* mice and islet hypertrophy and hyperplasia, and B-cell hyperplasia, are less severe, but B-cell degranulation is greater and degenerative islet lesions develop prematurely[5, 16]. Hyperplasia of the A-cells (alpha$_2$ glucagon-secreting cells) has been consistently observed in *ob/ob* mice[5, 21, 25, 30]. However, the B-cell hyperplasia is proportionately much greater than A-cell hyperplasia on the BL/6 and other backgrounds, but not on the BL/Ks background[5, 21, 25, 30].

Since the expression of the syndrome is modified by the background genome, it is important to establish the characteristic features of *ob/ob* mice on each background[16, 33]. This paper describes the *ob/ob* mice from the colony maintained at the University of Aston in Birmingham where the *ob* gene is carried on a mixed background. Mice from this colony or from the same stock are used widely for metabolic studies in the United Kingdom[1, 22, 33, 50, 57].

Previous studies of islet cell populations in *ob/ob* mice have not made a detailed examination of changes that occur at different ages[5, 8, 9, 21, 25, 30, 31, 56]. The present study includes a quantitative account of the B-cell and A-cell populations in Aston *ob/ob* mice between five and 40 weeks of age, and evaluates the changes in relation to plasma concentrations of insulin and glucagon.

Materials and methods

Obese hyperglycaemic mice (gene type *ob/ob*) and homozygous lean (*+/+*) mice from the colony maintained at the University of Aston in Birmingham were used. The origin of this colony is described in detail elsewhere[22]. Briefly, heterozygous C57BL/6J *ob/+* breeding pairs from the Jackson Laboratory, Bar Harbor, Maine, were obtained by the Institute of Animal Genetics, University of Edinburgh in 1957 and out-crossed to two non-inbred local strains: JH for higher litter size and CRL for higher growth rate[19, 20]. Heterozygous breeding pairs from this stock were obtained by the University of Aston in 1966 where they have been maintained in a closed non-inbred colony.

Mice that were used in this study were housed in an air-conditioned room at 22 ±2 °C, with a lighting schedule of 9.5 hours light (0800-1730) and 14.5 hours dark. A stardard pellet diet (Mouse breeding diet, Heygate & Sons Ltd., Northampton) and water were supplied *ad libitum*. Food intake was measured over four consecutive periods of 24 hours, as described previously[6]. Blood samples were obtained from the cut tip of the tail at 0900 hours for the determination of plasma glucose (10 µl), insulin (20 µl) and C-terminal immunoreactive glucagon (C-GLI) (100 µl). The plasma C-GLI determinations were performed on individual samples from separate groups of mice to those used for plasma glucose and insulin determinations. Different batches of identically reared mice were studied at each age. Equal numbers of male and female mice were used in each group. No significant differences ($P > 0.05$) were observed in the plasma parameters of males and females.

Plasma glucose was measured by an automated glucose oxidase procedure (Beckman Glucose Analyzer, Beckman Riic Ltd., High Wycombe)[48]. Plasma insulin was measured by dextran-charcoal radioimmunoassay[2] using crystalline mouse insulin as standard (Novo Industria A/S, Copenhagen). Plasma for C-GLI analysis was immediately extracted by a micro-modification of the method of

Heding[28] using a 60 per cent (v/v) final concentration of ice-cold ethanol. The procedure gave more than 97 per cent recovery of physiological amounts of glucagon. Analysis of C-GLI in the reconstituted plasma extracts was performed by dextran-charcoal radioimmunoassay[13] using monoiodotyrosine labelled ^{125}I porcine glucagon[36]. Crystalline porcine glucagon (lot 69/194), provided by the WHO International Laboratory for Biological Standards, Hampstead, London, was used as standard[4]. The antiserum (YY89) reacts with C-terminal fragments of the glucagon molecule (residues 18-29) and shows less than 2 per cent cross-reaction with gut glucagon-like immunoreactive material[12].

Histological studies were performed on tissue obtained from groups of four *ob/ob* and four *+/+* mice killed at intervals throughout the range five - 40 weeks, at the ages shown in Fig. 2 and the Table. Excised pancrea were fixed in 10 per cent formalin for 24 hours, dehydrated through graded alcohols, cleared in chloroform and embedded in paraffin wax. Consecutive sections of 5 μ thickness were stained with haematoxylin and eosin to demonstrate general islet morphology, aldehyde fuchsin[26] counterstained with 2 per cent aqueous light green to demonstrate B-cells, the silver impregnation method of Grimelius[27] to demonstrate A-cells, and Heidenhain's azan to demonstrate connective tissue. B-cells and A-cells were counted at X400 magnification using a squared graticule eyepiece. A mm^2 area of pancreas was examined on 10 sections from each of the four mice in each group. The sections were selected from the head through to the tail of the pancreas. The numbers of B-cells and A-cells were expressed as cells per mm^2 area of pancreas section. The area of individual islets, and the area occupied by vacuoles and acinar-like cells within the islets, were determined using a squared graticule eyepiece. The area occupied by vacuoles and acinar-like cells within the islets was expressed as a percentage of the total area of the islets.

Groups of data were compared using Student's *t*-test. Differences were considered significant for $P<0.05$.

Results

Lean mice

As shown in Fig. 1, the body weight of *+/+* mice increased slowly between five and 40 weeks, but food intake, and plasma concentrations of glucose, insulin and C-GLI were not significantly altered. The numbers of B-cells and A-cells (Fig. 2) were not significantly altered over the age range studied, but the B:A cell ratio was increased at 20 and 40 weeks compared with five weeks. The B-cells were consistently highly gradulated with aldehyde fuchsin positive staining material, and the normal localisation of B-cells (towards the interior of the islets) and A-cells (at the periphery of the islets) was maintained throughout.

Obese mice

Obesity was well established in *ob/ob* mice at five weeks of age and advanced until 30 weeks, after which the body weight declined (Fig. 1). The study was not extended beyond 40 weeks because most *ob/ob* mice die at about this age. Food intake of *ob/ob* mice was elevated between five and 20 weeks, but *ob/ob* mice consumed similar quantities of food to the *+/+* mice at 40 weeks. Plasma glucose concentrations were not significantly raised above those of *+/+* mice at five weeks,

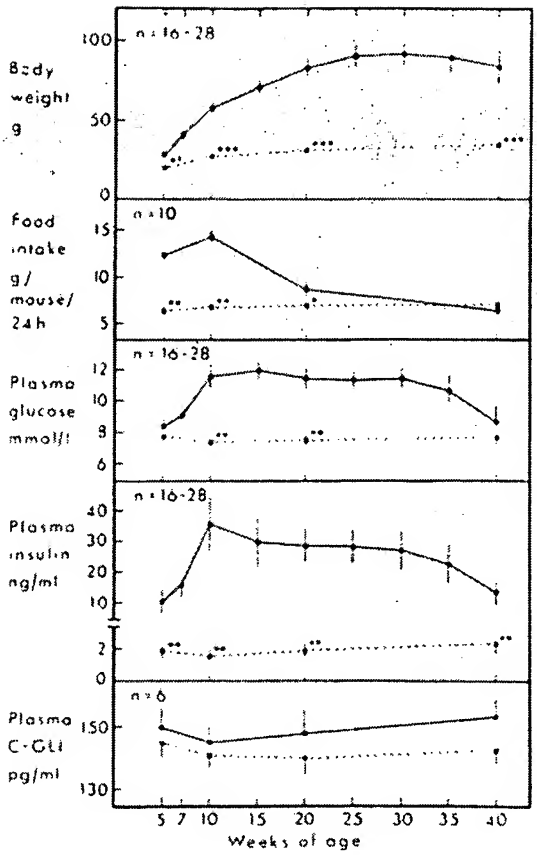

Fig. 1. Age-related changes in body weight, food intake and plasma concentrations of glucose, insulin and C-terminal immunoreactive glucagon (C-GLI) in freely fed obese *(ob/ob)* (● —— ●) and lean *(+/+)* (■ ··· ■) mice from the Aston colony. Values are mean ± s.e.m. *n*=number of determinations. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with *ob/ob* mice of the same age. +$P<0.05$ compared with 10, 20 and 40 week-old *(+/+)* mice

but a moderate hyperglycaemia was observed between seven and 35 weeks. Obese mice were hyperinsulinaemic at five weeks and plasma insulin concentrations rose markedly at ten weeks. The plasma insulin concentration then plateaued, and declined after 30 weeks. Plasma C-GLI concentrations were similar to those of *+/+* mice and did not vary significantly with age.

The islets of *ob/ob* mice showed B-cell hyperplasia at five weeks: the number of B-cells was more than three times greater than *+/+* mice (Fig. 2). The islets were hypertrophied and more richly vascularised than *+/+* mice at this age, but the B-cells were not as richly granulated with aldehyde fuchsin positive staining material. At five weeks there was marked A-cell hyperplasia: the number of A-cells was more than four times that of *+/+* mice. At this age the localisation of

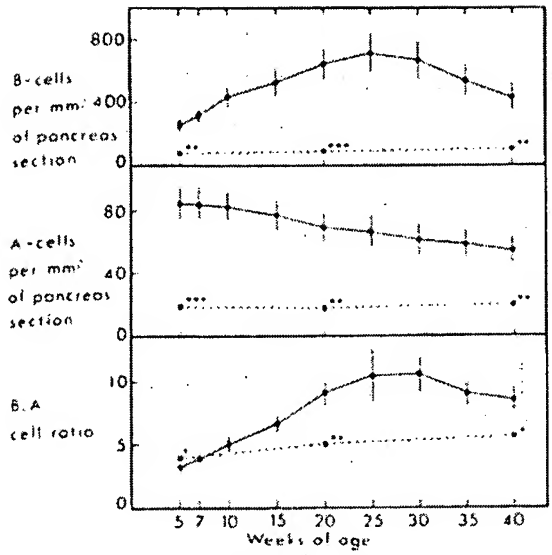

Fig. 2. Age-related changes in the numbers of B-cells and A-cells, and the B:A cell ratio in the islets of obese *(ob/ob)* (● ——— ●) and lean *(+/+)* (■ · · · · · ■) mice from the Aston colony. Values are mean ± s.e.m. of 40 determinations made from ten histological sections of pancreas from each of 4 mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with *ob/ob* mice of the same age. $^{+}P<0.05$ compared with 20 and 40 week-old *+/+* mice

B-cells and A-cells within the islets was normal (B-cells towards the interior and A-cells at the periphery).

B-cell hyperplasia in association with islet hypertrophy and hyperplasia increased until 25 weeks of age, when the B-cell population was seven times that of *+/+* mice (Fig. 2). Beyond this age the B-cell population declined. The B-cells showed very little granulation with aldehyde fuchsin positive staining material at ten weeks, but regranulation was observed in older *ob/ob* mice, although a state of full granulation comparable with *+/+* mice was not achieved. A fall in A-cell population was observed throughout life, but the number of A-cells was always significantly greater than in *+/+* mice (Fig. 2). As the *ob/ob* syndrome developed, the extent of B-cell hyperplasia exceeded that of the A-cells to produce a significant increase in the B:A cell ratio.

At ten weeks of age some of the larger islets contained small intercellular vacuoles. The vacuoles occupied less than 5 per cent of the area of the islets at this age (Table). After 25 weeks the size and number increased considerably, and an uncharacterised material was generally observed within the vacuoles, as shown in Fig. 3. The distribution of vacuoles was variable: some islets (mostly the larger ones) became extensively vacuolated while others (mostly the smaller ones) showed little or no vacuolation. Enlarging vacuoles coalesced, and a distinct cellular layer was frequently observed around the vacuole perimeter, giving a duct-like appearance (Fig. 3). The increasing vacuolation after 25 weeks was coincident with a decline in the B-cell population, and little futher islet

Table. The incidence of vacuolation and acinar-like cell inclusions in the islets of obese(*ob/ob*) mice from the Aston colony. Values are mean ± s.e.m. of 40 determinations made from ten histological sections of pancreas from each of four mice. Note, no vacuoles or acinar-like cell inclusions were observed in the islets of lean(*+/+*)mice

Age (weeks)	Islet vacuolation (percentage area of islets occupied by vacuoles)	Islet acinar-like cells (percentage area of islets occupied by acinar-like cells)
5	–*	–
7	–	–
10	< 5+	–
15	< 5	–
20	< 5	–
25	< 5	–
30	6.5 ± 1.5	–
35	14.0 ± 4.0	< 5
40	29.1 ± 7.2	20.5 ± 4.9

*–, not detectable

+ values less than 5 per cent could not be accurately quantitated

enlargement was apparent after this age. By 40 weeks, vacuoles accounted for 29 per cent of the area of the islets.

Serial sections of whole islets showed that a 25 weeks, and beyond, A-cells were often dispered throughout the interior of the islets (Fig. 4). The onset of capsular fibrosis was also apparent at this time and by 40 weeks there was considerable capsular and intra-islet fibrosis. Pancreatic acinar-like cells were occasionally observed within the islets at 35 weeks (Fig. 5). At this age the acinar-like cells comprised less than 5 per cent of the area of the islets (Table). This incidence of acinar-like cells within the islets increased by 40 weeks to occupy 20 per cent of the area of the islets. The acinar-like cells were generally arranged in clusters resembling acini, and appeared only in islets with considerab vacuolation. Serial sections of whole islets failed to reveal any disruption of the islet capsule, suggesting that a direct infiltration of the surrounding pancreatic exocrine tissue was not responsible. Occasionally islets became completely filled with acinar-like cells, leaving only scattered endocrine cells, remnants of vacuoles and the islet capsule as evidence of the islet's existence.

Discussion

The Aston *ob/ob* mouse (also referred to in the literature as the Birmingham *ob/ob* mouse) shows a unique expression of the obese hyperglycaemic syndrome. Maximal body weight is greater than in other colonies of *ob/ob* mice, possibly reflecting the trait for higher growth rate (CRL). Although a mild or moderate hyperglycaemia was observed at 0900 hours, a circadian study has shown that plasma glucose concentrations are higher at other times of the diurnal cycle[6]. Thus the hyperglycaemia of Aston *ob/ob* mice is intermediate between that of BL/6 *ob/ob* and BL/Ks *ob/ob* mice[10]. The severe hyperinsulinaemia, marked B-cell hyperplasia, islet hypertrophy and hyperplasia are characteristic of the

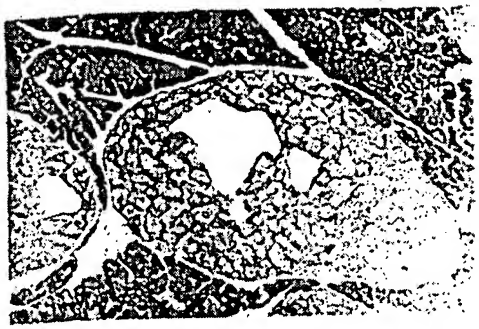

Fig. 3. Pancreas of 30 week-old obese *(ob/ob)* mouse showing islet with extensive vacuolation. Note the presence of material within the vacuoles and the appearance of a cellular layer around the vacuole perimeter. Haematoxylin and eosin, x 150 magnification

Fig. 4. Pancreas of 30 week-old obese *(ob/ob)* mouse showing islet with A-cells (densely stained) dispersed throughout the interior. Grimelius silver stain, x 200 magnification

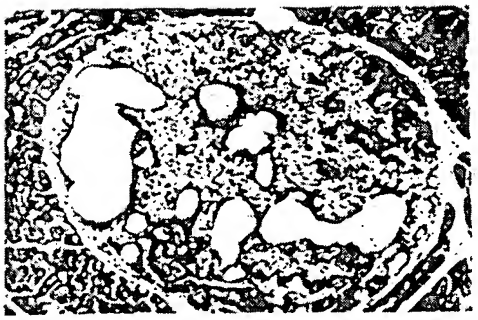

Fig. 5. Pancreas of 40 week-old obese *(ob/ob)* mouse showing islet with extensive vacuolation and acinar-like cells within the islet. Note distinct cellular layer around vacuoles giving a duct-like appearance. The acinar-like cells within the islets are arranged in clusters resembling acini. The islet capsule is thick and fibrotic. Haematoxylin and eosin, x 250 magnification

ob gene on the BL/6 background[5, 16], and in colonies originating directly from the Jackson V stock[31, 55]. However, the short life span, premature islet degeneration, relocation of A-cells to the islet interior, and the appearance of the vacuoles and acinar-like cells within the islets are reminiscent of BL/Ks *ob/ob* mice[5, 16].

The changes in islet structure and function in Aston *ob/ob* mice are consistent with the interpretation that increased insulin demand is associated with over-activity and neogenesis of B-cells[15]. Whether this situation precipitates a state of general B-cell exhaustion is uncertain, since freely fed 20-week-old *ob/ob* mice show an impaired insulin response to glucose[23], but an enhanced response to other secretagogues such as arginine and glucagon (Flatt

and Bailey, unpublished observations). The B-cell population was maximal at 25 weeks of age, when the number of B-cells was more than ten times greater than A-cells. Beyond this age the number of B-cells progressively declined, indicating that the rate of B-cell loss exceeded B-cell formation. This may result from increased B-cell death and degeneration. However, it is tempting to speculate that the B-cells lose their ability to recognise or respond to the mitogenic stimulus of glucose or other agents[29]. Indeed, the B-cells of ageing *ob/ob* mice may have completed their genetically determined mitotic potential[29,40]. The A-cell hyperplasia of *ob/ob* mice was greatest at five weeks, suggesting that an increase in the number of A-cells is an early feature of this syndrome. An explanation for the relocation of A-cells within the interior of the islets of ageing *ob/ob* mice is unclear. This may be related to the decline in B-cell numbers, since a similar change can be induced by B-cell cytotoxic agents such as streptozotocin and alloxan[10,47,54]. The internalisation of A-cells has also been observed within the islets of several genetically transmitted animal syndromes of obesity and diabetes[5,37], and follows B-cell destruction in human juvenile type diabetes[52].

The origin of the islet vacuoles in *ob/ob* mice is unknown. It is interesting to note that similar vacuoles developed when islets of *ob/ob* mice from the Jackson V stock were cultured for seven days in media containing a high (16.7 mmol/l) glucose concentration[3]. The superficial resemblance of these vacuoles to the insular hyalinosis in elderly human diabetics[24,53], and the possibility of a similar pathogenesis, deserves investigation. Since there was no evidence of exocrine invasion of the islets, the acinar-like cells within the islets of ageing *ob/ob* mice might result from endocrine-exocrine transformation, as suggested for similar inclusions within the islets of other strains and species[35,39,42,44-46].

Although the early development of obesity was associated with hyperphagia, maximal body weight coincided with reduction of food intake to that of *+/+* mice. This suggests that low energy expenditure, due to inactivity[11], reduced thermogenesis in brown adipose tissue[49,50] and increased efficiency of fuel utilization[11], make an important contribution to the obesity in these animals. The present study illustrates the close relationship between hyperphagia and hyperinsulinaemia in young *ob/ob* mice, and confirms that these features precede the onset of hyperglycaemia[11,17,33]. The observation that food intake and plasma insulin concentrations fall considerably at 40 weeks, supports the view that hyperalimentation and increased activity of the entero-insular axis are key factors in the genesis and maintenance of hyperinsulinaemia at earlier stages of the syndrome[7,43]. The fall in plasma glucose concentrations at 40 weeks may be attributed to the reduction in food intake and the markedly improved insulin sensitivity in *ob/ob* mice at this age (Flatt & Bailey, unpublished observations).

Although the *ob/ob* mice displayed marked A-cell hyperplasia which declined with advancing age, plasma C-GLI concentrations were similar to those of *+/+* mice and did not vary significantly with age. Since glucagon is normally suppressed by high concentrations of insulin and glucose[51] the plasma C-GLI concentrations of *ob/ob* mice can be regarded as paradoxically high. Inappropriate hyperglucagonaemia has been noted in BL/6 *ob/ob* mice[18,38,41]. Preliminary studies in Aston *ob/ob* mice indicate that the paradoxically high glucagon concentrations result in part from insensitivity of the A-cells to the inhibitory

influence of insulin[23].

In conclusion, Aston *ob/ob* mice display a unique combination of different features shown by *ob/ob* mice on the BL/6, BL/Ks and other backgrounds. The present study emphasises that experiments using *ob/ob* mice should define clearly the age as well as the genetic background[16, 33].

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